TRADE SECRET

Study Title

H-27529:
In Vitro Mammalian Chromosome Aberration Test in Chinese Hamster Ovary Cells

TEST GUIDELINES: U.S. EPA Health Effects Test Guidelines
OPPTS 870.5375 (1998)

OECD Guidelines for the Testing of Chemicals
Section 4 (Part 473) (1998)

EC Commission Directive 2000/32/EC Annex 4A-B10
Number L 136

ORIGINAL REPORT AUTHOR: Christine M. Glatt, M.S.

REPORT REVISION 1 AUTHOR: Kyle P. Glover, B.A.

ORIGINAL REPORT COMPLETED: June 27, 2006

REPORT REVISION 1 COMPLETED: February 25, 2008

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
DuPont Haskell Global Centers for
Health & Environmental Sciences
P.O. Box 50
Newark, Delaware 19714
U.S.A.

LABORATORY PROJECT ID: DuPont-19714

WORK REQUEST NUMBER: 16540

SERVICE CODE NUMBER: 531

SPONSOR: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are compatible with current OECD and MAFF (Japan) Good Laboratory Practices, except for the items documented below. None of the items listed impact the validity of the study.

1. At the request of the sponsor, this study was conducted using test substance that was not characterized.

2. Neither the vehicle nor the positive controls were characterized by the testing facility or the sponsor. However, both the vehicle and positive controls were purchased from a reputable vendor and showed results consistent with historical control data.

3. The concentrations of the positive control and test substance dose solutions were not confirmed analytically; however, the solutions were prepared by trained personnel to ensure the accuracy of the concentrations.

Study Director: _____ 25-FEB-2008

Kyle P. Glover, B.A.
Associate Scientist

Date

QUALITY ASSURANCE STATEMENT

Work Request Number: 16540
Study Code Number: 531

Phase Audited	***Audit Dates***	***Date Reported to Study Director***	***Date Reported to Management***
Protocol:	March 17, 2006	March 17, 2006	March 20, 2006
Conduct:	March 28, 2006	March 28, 2006	March 28, 2006
Report/Records:	May 13-16, 2006	June 21, 2006	June 26, 2006
Report Revision 1:	February 21, 2008	February 21, 2008	February 21, 2008

Reported by: for Donna M. Johnston 25 February 2008
Donna M. Johnston
Quality Assurance Auditor
Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Reviewed and Approved by: E. Maria Donner — 25-Feb-2008
E. Maria Donner, Ph.D.
Senior Research Toxicologist and Manager
Date

Issued by Study Director: — 25-FEB-2008
Kyle P. Glover, B.A.
Associate Scientist
Date

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF APPENDICES

Page

STUDY INFORMATION

Substance Tested:	• Crude Industrial Grade HFPODA Ammonium Salt • H-27529
Haskell Number:	27529
Composition:	85.4-85.8 wt% Balance is water
Purity:	See composition, above
Physical Characteristics:	Clear liquid
Stability:	The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.
Study Initiated/Completed:	March 16, 2006 / (see report cover page)
Experimental Start/Termination:	March 21, 2006 / April 6, 2006

REASON FOR REVISION 1

The name of the substance tested was revised on the Study Information Page.

SUMMARY

The test substance, H-27529, was evaluated for its ability to induce chromosome aberrations *in vitro* in Chinese hamster ovary (CHO) cells in both the presence and absence of an exogenous S9 metabolic activation system. To establish a concentration range for the chromosome aberration assay, a preliminary toxicity assay was initially conducted.

The test substance was prepared in sterile water as this vehicle was determined to be the solvent of choice based on solubility of the test substance and compatibility with the target cells. The test substance was soluble in the vehicle at approximately 50 mg/mL (144 mM). The highest stock concentration that was prepared for use on the study was 34.71 mg/mL (100 mM). The dosing solution concentrations were adjusted to compensate for the purity of the test substance using a correction factor of 1.18.

In the preliminary toxicity and chromosome aberration assay, the cells were treated for 4 and 20 hours in the non-activated test condition and for 4 hours in the S9-activated test condition. All cells were harvested at 20 hours after treatment initiation. A vehicle control group was included in each test condition.

In the preliminary toxicity assay, the highest concentration tested was 3471 µg/mL (10 mM), the limit dose according to the guideline for this test (i.e., 5000 µg/mL or 10 mM, whichever is lower). The osmolality of the highest test substance concentration in medium was similar to the vehicle control. The test substance was soluble in the vehicle and in the treatment medium at all concentration levels tested. The pH of the highest test substance concentration in medium was similar to the vehicle control and did not change throughout the test. No test substance precipitation was observed. Substantial toxicity (at least a 50% reduction in cell growth relative to the vehicle control) was observed at the highest concentration level in the 20-hour non-activated test condition only. Based on the findings from the preliminary toxicity assay, the highest concentration chosen for the chromosome aberration assay was 3471 µg/mL (10 mM) for all three test conditions.

The concentrations chosen for the chromosome aberration assay were 100, 500, 1000, 2500, and 3471 µg/mL for all three test conditions. The difference in osmolality between the highest test substance concentration in medium and the vehicle in medium was less than 20%. The pH of the highest test substance concentration in medium was similar to the vehicle control and did not change throughout the test. No test substance precipitation was observed. No substantial toxicity (at least 50% reduction in the cell growth relative to the vehicle control) was observed in either 4-hour test condition. Substantial toxicity was observed in the 20-hour non-activated test condition at 3471 µg/mL (58% reduction). Additionally, a substantial reduction in the mitotic index relative to the vehicle control was observed in the 20-hour non-activated test condition at 1000 µg/mL (63.8% reduction).

Cytogenetic evaluations were conducted at 1000, 2500, and 3471 µg/mL for the 4-hour non-activated and 4-hour S9-activated test conditions and at 100, 500, and 1000 µg/mL for the 20-hour non-activated test condition. These concentrations were chosen based on cell growth reduction and mitotic index inhibition. The percentage of cells with structural aberrations in the test substance-treated groups was not increased above that of the vehicle control at any concentration. The percentage of cells with numerical aberrations at 3471 µg/mL in the 4-hour non-activated and at 2500 and 3471 µg/mL in the 4-hour S9-activated test conditions was increased above that of the vehicle control ($p < 0.05$, Fisher's exact test). These observed changes were dose-dependent ($p < 0.05$, Cochran-Armitage test), were outside the historical control range of 0-5% (non-activated) and 0-5% (S9-activated) for numerical aberrations, and were considered biologically significant. The observed numerical aberrations were primarily in the form of endoreduplication.

All criteria for a valid study were met. Under the conditions of this study, H-27529 was not found to induce structural chromosome aberrations in the *in vitro* mammalian chromosome aberration test in Chinese hamster ovary cells. However, it was found to induce numerical chromosome aberrations in both the non-activated and S9-activated test systems at 4 hours. It was concluded that the test substance was negative in this *in vitro* test.

INTRODUCTION

The objectives of this study were to evaluate the test substance, H-27529, for its ability to induce structural chromosome aberrations in Chinese hamster ovary (CHO) cells *in vitro*. Numerical aberrations were recorded. The assessment was done both in the presence and absence of an exogenous S9 metabolic activation system.

MATERIALS AND METHODS

A. Testing Guidelines

This study was conducted in compliance with the following guidelines:

- U.S. EPA, OPPTS 870.5375: *In Vitro* Mammalian Chromosome Aberration Test, *Health Effects Test Guidelines* (1998)
- Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, *In Vitro* Mammalian Chromosome Aberration Test, Number 473 (1998)
- European Commission Directive 2000/32/EC of May 19, 2000, Annex 4A-B10: Mutagenicity - *In Vitro* Mammalian Chromosome Aberration Test, Number L 136.

B. Test Substance and Controls

1. Identification

The test substance, H-27529, was a clear liquid. The test substance batch used for this study was assigned Haskell Identification Number 27529. Additional information regarding the test substance is found on the study information page of this report.

2. Sample Preparation, Stability, and Analytical Verification of Test Substance Concentrations

The sponsor-reported purity for H-27529 was 85.4-85.8% active ingredient. A correction factor (1.18) based on the percent active ingredient was used for preparation of the dosing solutions and, therefore, all doses are reported as µg active ingredient per mL. An analytical verification of the test substance concentrations was not conducted.

3. Controls

Negative:	Sterile Water (CAS# 7732-18-5, molecular grade, Mediatech Inc.)
Positive:	Mitomycin C (MMC, CAS# 50-07-7, Sigma) Cyclophosphamide (CP, CAS# 6055-19-2, Sigma)

The positive controls were dissolved in sterile water. The positive controls were assumed to be stable during this assay and no evidence of instability was observed.

C. Test System

The CHO-K_1 cell line was originally derived as a subclone from a parental CHO cell line. The cells require proline in the medium for growth, and have a modal chromosome number of 20. The population doubling time is 10-14 hours. The cell line was obtained from the American Type Culture Collection (ATCC number CCL 61), Manassas, Virginia. The karyotype and the absence of mycoplasm infection are routinely checked by Haskell Laboratory. This test system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.[1]

D. Experimental Design and Methodology

The study was conducted according to published procedures.[1-5] The test substance, as well as positive and negative (vehicle) controls, was administered in the presence and absence of an exogenous S9 metabolic activation system to cell cultures by addition to the culture medium. In the non-activated test system, the treatment times were approximately 4 and 20 hours, and in the S9-activated test system, approximately 4 hours. The dividing cells were arrested in metaphase approximately 18 hours after initiation of the treatment and harvested at approximately 20 hours. This harvest timepoint represents approximately 1.5 normal cell cycles, and is determined to ensure assessment of clastogenicity in first-division metaphase cells.[4] Cytogenetic analyses were conducted on the 4- and 20-hour non-activated and 4-hour activated assays. The cytogenetic assessment also included recording of numerical aberrations. Based on OECD 473, a clear positive response does not require verification. Negative results do not require confirmation, but are justified. Equivocal results may need to be confirmed, and may require a modified study design.

1. Solubility Determination and Selection of Vehicle

A solubility determination was conducted to determine the maximum soluble concentration of a workable suspension up to a maximum of 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles. Vehicles compatible with this test system, in order of preference, included, but were not limited to, culture medium or sterile water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), or ethanol (CAS 64-17-5). The vehicle of choice for this study was sterile water, which permitted preparation of the highest workable/soluble stock concentration. Under the conditions of this test system, the final concentration of solvents other than water, physiological buffer, or medium did not exceed 10% of the treatment medium. After the addition of the dosing solution, the treatment medium was observed for precipitation (with the naked eye).

2. Exogenous Metabolic Activation

Liver homogenate (S9), prepared from male Sprague-Dawley rats induced with Aroclor 1254, was purchased commercially (Moltox, Inc., Boone, NC) and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 liver homogenate was thawed and mixed with a cofactor pool. The final concentration of the cofactors and S9 in the metabolic activation system (S9 mixture) was 4 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer, pH 7.4, and 10% S9. The S9

mixture was prepared immediately before use and kept on ice until used. The metabolic activity of the S9 was demonstrated by the response of the CP-treated cultures.

3. Flask Identification

Using computer generated labels or a permanent marker, each flask or tube was labeled with the work request number, the Haskell number, dose level, replicate indicator (A or B), metabolic activation system (+/-S9), exposure period, and date.

4. Frequency and Route of Administration of the Dosing Solutions

Cell cultures were treated once for approximately 4 hours in the absence and presence of S9 metabolic activation, and for approximately 20 hours in the absence of metabolic activation. The test substance was added to the treatment medium in a test system-compatible vehicle. This frequency and route of administration has been demonstrated to be effective in the detection of chemically-induced mutagenesis in this test system.[2-3]

5. Preparation of Target Cells for the Preliminary Toxicity Assay

Exponentially growing CHO-K_1 cells were seeded in labeled, sterile flasks. Approximately 5×10^5 cells/25 cm^2 flask were inoculated in complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units penicillin/mL and 100 µg streptomycin/mL). Cultures were incubated at 37 ± 2°C in a humidified atmosphere of 5 ± 2% CO_2 in air for 16-24 hours.

6. Preliminary Toxicity Assay to Select Dose Levels

The selection of dose levels for the cytogenetics assay was based on a preliminary toxicity assay. At the start of the assay, cell cultures seeded 16-24 hours earlier (one culture per concentration level) were exposed to 9 concentrations of the test substance and the vehicle control substance. The day when the cells were first exposed to the test substance was designated as test day 0. The dividing cells were harvested at a single timepoint, approximately 20 hours after the initiation of treatment (~1.5 times the normal cell cycle). The exposure times were approximately 4 and 20 hours in the absence of S9 metabolic activation, and approximately 4 hours in the presence of S9 metabolic activation. After the 4-hour exposure period only, the treatment medium was removed and replaced with complete McCoy's 5A culture medium. Approximately 20 hours after the initiation of exposure to the test substance, the cell cultures were microscopically inspected for the extent of monolayer confluency relative to the vehicle control. The cells were also harvested by trypsinization and counted by an automatic cell counter. Cell viability data were obtained, but not reported. The cell counts were used to determine cell growth inhibition relative to the solvent control.

The osmolality and pH of the vehicle control, as well as the highest soluble test substance concentration in the culture media, was determined. An increase of ≤20% in the osmolality of the test substance concentration was considered acceptable. The pH of the treatment medium was evaluated both at the beginning and the end of the treatment period by visual determination using the pH-sensitive color dye present in the treatment media. A visual inspection of the

treatment medium for precipitation was also conducted at the beginning and end of the treatment period.

Except for the highest concentration in the 20-hour non-activated test condition (59% cell growth inhibition), none of the concentrations that were tested in the preliminary toxicity assay induced greater than a 50% cell growth inhibition relative to the vehicle control. Furthermore, no precipitation was observed at any concentration tested in the preliminary toxicity assay. Therefore, the highest concentration that was selected for the chromosome aberration assay was the limit dose of 3471 µg/mL (10 mM).

Whenever possible, the highest concentration selected for the chromosome aberration assay induced greater than a 50% cell growth inhibition relative to the vehicle control. At least two additional dose levels, demonstrating limited toxicity or no toxicity were also evaluated. In cases where there was little or no cytotoxicity, the highest dose level tested and at least two lower dose levels were selected for analysis. In cases where there was little or no cytotoxicity, but a precipitate in the treatment medium was observed (with the naked eye), the lowest dose level demonstrating a precipitate and two other lower dose levels were selected for analysis.

If neither cytotoxicity nor precipitation was observed in the preliminary toxicity assay, the highest concentration was the limit dose of 5000 µg/mL or 10 mM, whichever was the lower.

7. Chromosome Aberration Assay

a. Preparation of Target Cells for the Chromosome Aberration Assay

Exponentially growing CHO-K_1 cells were seeded in labeled, sterile flasks. Approximately 5×10^5 cells/25 cm^2 flask were inoculated in complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units penicillin/mL and 100 µg streptomycin/mL). Cultures were incubated at 37 ± 2°C in a humidified atmosphere of 5 ± 2% CO_2 in air for 16-24 hours.

b. Negative Control

The test substance vehicle, sterile water, was used as the concurrent negative control. The final concentration of vehicle in the treatment medium did not exceed 10%.

c. Positive Control

The positive control substances were mitomycin-C (MMC) for the non-activated system and cyclophosphamide (CP) for the S9-activated system. The concentrations for MMC were 0.2 and 0.4 µg/mL, and for CP, 5 and 10 µg/mL. Both positive control substances were dissolved in sterile water. Two test concentrations of each positive control substance were used to ensure a valid assay; however, only one of the concentrations of each positive control was included in the cytogenetic analysis. The exposure periods for MMC were approximately 4 and 20 hours, and the exposure period for CP was approximately 4 hours. Exposure to the positive control substances was included in the chromosome aberration assay, but not in the preliminary cytotoxicity portion of the study. The positive controls were not expected to contain any contaminants that would interfere with the conduct of the study, and were expected to be stable

under the conditions of administration. Concentration verification of the positive control substances was not conducted. No positive control substances were used for numerical aberrations (polyploidy or endoreduplication, or both).

d. Treatment of Target Cells

The day when the cells were first exposed to the test substance was designated as test day 0. Sixteen to twenty-four hours after seeding the CHO cultures, the culture medium was discarded and replaced with approximately 5 mL complete medium for the non-activated test condition, and 4 mL complete medium plus 1 mL of the S9 mixture for the activated test condition. The volumes were selected such that addition of the test substance volume (0.5 mL) resulted in a total volume of approximately 5 mL.

Sets of duplicate cultures were then administered an aliquot of the test substance (at least five concentrations were applied), the vehicle control, or two positive control substance concentrations for each test condition. The treatment medium was McCoy's medium for all test conditions. The cells were treated for approximately 4 and 20 hours in the non-activated test conditions, and for approximately 4 hours in the S9-activated test condition. After completion of the 4-hour exposure periods only, the cells were collected by centrifugation, washed once with phosphate buffered saline, fed with complete medium, and incubated until cell harvest. The incubations were conducted at 37 ± 2°C in a humidified atmosphere of 5 ± 2% CO_2 in air.

An evaluation of all cultures was made at the beginning and end of the treatment period by visual determination to assess both pH and precipitation.

e. Collection of Metaphase Cells and Cell Harvest

The cells were arrested in metaphase approximately 18 hours after treatment initiation by adding Colcemid® to the cultures at a 0.1 µg/mL final concentration in the culture media. Approximately 20 hours after treatment initiation, the cultures were washed with PBS, trypsinized, collected by centrifugation, and the cells were resuspended in 5 mL fresh medium. A concurrent cytotoxicity measurement determining total cell growth inhibition (%) relative to the solvent control was conducted for all assays and test conditions using an automated cell counter. In addition, a physical examination of cell growth (monolayers cell confluency) was conducted for all test conditions. The cells were treated with 0.075M KCl hypotonic buffer, fixed once in methanol and 3 times in methanol:glacial acetic acid (3:1 v/v), and stored frozen overnight. To prepare slides, the cells were collected by centrifugation and resuspended in fresh fixative. At least two slides per culture were prepared by applying an aliquot of the fixed cells onto clean microscope slides and air-drying them. The slides were stained by Giemsa and permanently mounted.

8. Identification of the Slides

The slides were identified by the work request number, the Haskell number, dose level, replicate indicator (if applicable, i.e., A, B, C, etc.), metabolic activation system (+/-S9), exposure period (4 or 20 hours), and date.

9. Cytogenetic Analyses

Cytogenetic analyses were conducted for at least three test substance concentrations, the vehicle control, and the positive controls. The highest test substance concentration level that was analyzed was that which caused a cell growth inhibition of greater than 50% when compared to the vehicle control or, if the test substance lacked toxicity, the highest scorable concentration used in the test. The percentage of cells in metaphase per at least 1000 cells scored per concentration level (at least 500 from each duplicate culture) was determined prior to coding the slides. After selection of the slides for cytogenetic analyses, the slides were coded and scored. Metaphase cells were selected for scoring based on good chromosome morphology and staining characteristics. Only metaphase cells with 20 ± 2 centromeres were analyzed for structural aberrations. However, a structural aberration occurring in a polyploid cell was included in the analyses. At least 200 metaphases per concentration level (100 from each duplicate culture), when available, were analyzed for structural aberrations.[5] Numerical aberrations were recorded as well. The number of metaphases evaluated per duplicate flask was less if 10 or more aberrant cells were observed among the first 25 cells scored. Chromatid-type aberrations included chromatid and isochromatid breaks and exchange figures. Chromosome-type aberrations included chromosome breaks and exchange figures. Pulverized chromosome(s) and cells, and severely damaged cells (i.e., cells with ≥10 aberrations per cell) were recorded, but not included in the analyses. The XY coordinates for the microscope stage were recorded for cells with structural or numerical aberrations.

E. Criteria for Determination of a Valid Test

An assay was considered acceptable for evaluation of test results only if all of the following criteria were satisfied. The metabolically activated and non-activated assays of the test are independent and, if necessary, were repeated separately.

1. Negative Controls

The frequency of cells with structural chromosome aberrations was in the frequency range of the historical control vehicle.

2. Positive Controls

The percentage of cells with structural chromosome aberrations must be statistically significantly greater ($p \leq 0.05$, Fisher's exact test) than the vehicle control response.

F. Evaluation of Test Results and Statistical Analyses

The clastogenic potential of the test substance was assessed based on its ability to induce structural chromosome aberrations. The experimental unit is the cell; therefore the percentage of cells with structural aberrations was used for the assessment.

Data was evaluated using scientific judgment. Statistical analysis was used as a guide to determine whether or not the test substance induced a positive response. Interpretation of the statistical analysis also relied on additional considerations including the magnitude of the observed test substance response relative to the vehicle control response and the presence of a

dose responsive trend. Statistical analysis consisted of a Cochran-Armitage test for dose responsiveness and a Fisher's exact test to compare the percentage of cells with structural or numerical aberrations in the test substance treated groups with the vehicle control response.[6-7]

The following conditions were used as a guide to determine a positive response:

- A statistically significant increase ($p \leq 0.05$, Fisher's exact test) in the percentage of cells with structural aberrations was seen in one or more treatment groups relative to the vehicle control response.
- The observed increased frequencies were accompanied by a concentration-related increase.
- A statistically significant increase was observed at the highest dose only.
- Note: Statistically significant values that did not exceed the historical control range for the negative/vehicle control may be judged as not being biologically significant.

The following condition was used as a guide to determine an equivocal response:

- Results observed in any of the assays resulted in statistically significant elevations in structural chromosome aberrations at more than one test concentration level, except the highest dose, without demonstrating a dose-responsive trend.

The test substance was judged negative if the following condition was met:

- There was no statistically significant increase in the percentage of cells with structural aberrations in any treatment group relative to the vehicle control group.

G. Data Presentation

The data was summarized in tables containing cell counts, cell growth inhibition, mitotic index, percent polyploidy/endoreduplication (numerical aberrations), number of cells analyzed, types of structural aberrations, frequencies of structural aberrations per cell, and the percentage of cells with structural aberrations. Chromatid and chromosome gaps were listed, but not added to the totals for structural aberration evaluation (gaps are not considered true structural damage).

RESULTS AND DISCUSSION

A. Solubility

Sterile water was determined to be the solvent of choice based on the solubility of the test substance and compatibility with the target cells.[6] The test substance formed a clear solution in the vehicle at the highest stock concentration prepared, 34.71 mg/mL.

B. Preliminary Toxicity Assay

(Table 1)

Concentrations for the chromosome aberration assay were selected based on the results from a preliminary toxicity test assessing the cell growth inhibition relative to the vehicle control. The cultures were microscopically inspected for the extent of monolayer confluency relative to the vehicle control. The data are not included in the report; the assessment is mainly an aid for study conduct. In the preliminary toxicity assay, the highest concentration tested was 3471 µg/mL (10 mM), the limit dose according to the guideline for this test (i.e., 5000 µg/mL or 10 mM, whichever is lower). CHO cells were exposed to the vehicle control or a total of nine concentrations of the test substance ranging from 0.3 to 3471 µg/mL (10 mM). The cells were exposed for 4 hours in both the absence and presence of an exogenous metabolic activation system (Aroclor-induced S9), or for 20 hours in the absence of S9 activation. The test substance was soluble in the vehicle and in the treatment medium at all concentrations tested. No precipitation was observed at the beginning or end of the treatment periods. Based on visual inspection of the pH-sensitive treatment medium at the beginning and end of the treatment periods, the pH of the highest test substance concentration in media was similar to the pH of the vehicle control and the pH did not change during the treatment period. Osmolality and pH measurements were taken from the highest test substance concentration and the vehicle control media. The osmolality and pH of the highest test substance concentration was similar to that of the vehicle control. In the treatment medium of the highest concentration tested, the osmolality was 289 and 285 mmol/kg in the non-activated and activated test condition, respectively. The pH of the highest concentration in the treatment medium was 7.6 and 7.4 in the non-activated and activated test condition, respectively. The osmolality of the vehicle in the treatment medium was 269 and 268 mmol/kg in the non-activated and activated test condition, respectively. The pH of the vehicle in the treatment medium was 7.4 and 7.3 in the non-activated and activated test condition, respectively. Substantial toxicity (at least a 50% reduction in cell growth relative to the vehicle control) was observed at the highest concentration level in the 20-hour non-activated test condition only (Table 1). Based on the findings from the preliminary toxicity assay, the highest concentration chosen for the chromosome aberration assay was 3471 µg/mL (10 mM) for all three test conditions.

C. Chromosome Aberration Assay

(Tables 2-8)

Based on the findings from the preliminary toxicity assay, the concentrations chosen for the chromosome aberration assay were 100, 500, 1000, 2500, and 3471 µg/mL (10 mM) for all three test conditions. The osmolality and pH of the highest test substance concentration were similar to the osmolality and pH of the vehicle control. In the treatment medium of the highest concentration tested, 3471 µg/mL, the osmolality was 291 mmol/kg in both the non-activated and activated test conditions. The pH of the highest concentration in the treatment medium was 7.6 and 7.5 in the non-activated and activated test conditions, respectively. The osmolality of the vehicle in the treatment medium was 269 and 267 mmol/kg in the non-activated and activated test conditions, respectively. The pH of the vehicle in the treatment medium was 7.6 and 7.3 in the non-activated and activated test conditions, respectively. No precipitation was observed in

the treatment medium at any concentration level in any test condition at the beginning or end of treatment. In addition, the visual examination of the pH-sensitive medium did not change in pH from the beginning to the end of treatment. The cultures were also microscopically inspected for the extent of monolayers confluency relative to the vehicle control. These data are not included in the report; the assessment was mainly an aid for study conduct. In addition, the uncoded slides were microscopically scanned for the presence of mitotic cells to ensure the selection of scorable test substance concentrations (Tables 2-4).

The concurrent toxicity data are presented in Tables 2–4. Based on the concurrent toxicity assay and assessing the mitotic index, the 1000, 2500, and 3471 µg/mL concentrations were selected for chromosome aberration analyses for the 4-hour non-activated and 4-hour S9-activated test conditions. The mitotic index inhibition at 3471 µg/mL was 33.1% and 24.4% in the 4-hour non-activated and 4-hour S9-activated test conditions, respectively. In the 20-hour non-activated test condition, there was a 97.6% and 99% decrease in mitotic figures at the 2500 and 3471 µg/mL concentration levels, respectively. The mitotic index inhibition at 1000 µg/mL was 63.8%. Therefore, the 100, 500, and 1000 µg/mL concentrations were selected for chromosome aberration analyses for the 20-hour non-activated test condition.

The cytogenetic analysis findings from the individual treatment cultures in the non-activated 4-hour exposure group are presented in Table 5 and summarized by group in Table 8. At the highest test concentration evaluated microscopically for chromosome aberrations, 3471 µg/mL, a 32 % growth inhibition in relation to the vehicle control was observed (Table 2). The mitotic inhibition was 33.1% relative to the vehicle control. The percentage of cells with structural aberrations in the test substance-treated groups was not significantly increased above that of the vehicle control group at any concentration ($p > 0.05$, Fisher's exact test). The percentage of cells with structurally damaged chromosomes in the MMC (positive control) treatment group (38%) was statistically significant. The percentage of cells with numerical aberrations in the 3471 µg/mL group was significantly increased above that of the vehicle control group ($p < 0.05$, Fisher's exact test). These observed changes were dose-dependent ($p < 0.05$, Cochran-Armitage test), were outside the historical control range of 0-5% for numerical aberrations, and were considered biologically significant. The observed numerical aberrations were primarily in the form of endoreduplication.

The cytogenetic analysis findings from the individual treatment cultures in the S9-activated 4-hour exposure group are presented in Table 6 and summarized by group in Table 8. At the highest test concentration evaluated microscopically for chromosome aberrations, 3471 µg/mL, no growth inhibition in relation to the vehicle control was observed (Table 3). The mitotic inhibition was 24.4% relative to the vehicle control. The percentage of cells with structural aberrations in the test substance-treated groups was not significantly increased above that of the vehicle control group at any concentration ($p > 0.05$, Fisher's exact test). The percentage of cells with structurally damaged chromosomes in the CP (positive control) treatment group (16.5%) was statistically significant. The percentage of cells with numerical aberrations in the 2500 and 3471 µg/mL groups was significantly increased above that of the vehicle control group ($p < 0.05$, Fisher's exact test). These observed changes were dose-dependent ($p < 0.05$, Cochran-Armitage test), were outside the historical control range of 0-5% for numerical aberrations, and were considered biologically significant. The observed numerical aberrations were primarily in the form of endoreduplication.

The cytogenetic analysis findings from the individual treatment cultures in the non-activated 20-hour exposure group are presented in Table 7 and summarized by group in Table 8. At the highest test concentration evaluated microscopically for chromosome aberrations, 1000 µg/mL, a 28 % growth inhibition in relation to the vehicle control was observed (Table 4). The mitotic inhibition was 63.8% relative to the vehicle control. The percentage of cells with structural or numerical aberrations in the test substance-treated groups was not significantly increased above that of the vehicle control group at any concentration ($p > 0.05$, Fisher's exact test). The percentage of cells with structurally damaged chromosomes in the MMC (positive control) treatment group (40%) was statistically significant.

CONCLUSION

All criteria for a valid study were met. Under the conditions of this study, H-27529 was not found to induce structural chromosome aberrations in the *in vitro* mammalian chromosome aberration test in Chinese hamster ovary cells. However, it was found to induce numerical chromosome aberrations in both the non-activated and S9-activated test systems at 4 hours. It was concluded that the test substance was negative in this *in vitro* test.

RECORDS AND SAMPLE STORAGE

Specimens (if applicable), raw data, the protocol, amendments (if any), and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

REFERENCES

1. Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., and Wassom, J.S. (1981). Mammalian in vivo and in vitro cytogenetic assays: A report of the Gene-Tox Program, Mutation Research, 87:143-188.

2. Evans, H.J. (1976). Cytological Methods for Detecting Chemical Mutagens. In: Chemical Mutagens, Principles and Methods for their Detection, Vol. 4, Hollaender, A. (ed.), Plenum Press, New York and London.

3. Swierenga, S.H.H., Heddle, J.A., Sigal, E.A., Gilman, J.P.W., Brillinger, R.L., Douglas, G.R., and Nestmann, E.R. (1991). Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese lung and human lymphocyte cultures, Mutation Research 246:301-322.

4. Galloway, S.M., Aardema, M.J., Ishidate Jr., M., Ivett, J.L., Kirkland, D.J., Morita, T., Mosesso, P., and Sofuni, T. (1994). Report from working group on in vitro tests for chromosomal aberrations, Mutation Research 312(3):241-261.

5. Scott, D., Danford, N.D., Dean, B.J., and Kirkland, D.J. (1990). Metaphase Chromosome Aberration Assays In Vitro. In: Basic Mutagenicity Tests: UKEMS Recommended Procedures. D.J Kirkland (ed). Cambridge University Press, New York, NY.

6. Fisher, R.A. (1985). Statistical Methods for Research Workers, 13th edition. Haffner, New York.

7. Snedecor, G.W. and Cochran, W.G. (1967). Statistical Methods, 6th edition, pp 246-248 and 349-352. The Iowa State University Press, Iowa.

TABLES

TABLES

EXPLANATORY NOTES

ABBREVIATIONS:

% Aberrant Cells	Cells with numerical aberrations include polyploid and endoreduplicated cells; cells with structural aberrations exclude cells with only gaps
Aberrations Per Cell	Cells with severely damaged chromosomes or with 10 or more structural aberrations were counted as 10 aberrations
Br	break
Cell Growth Index	(cells per flask treated group/cells per flask control group), expressed as a percentage
Cell Growth Inhibition	(cell growth index control group – cell growth index treated group), expressed as a percentage
CHO	Chinese hamster ovary
Chromatid Breaks	include chromatid and isochromatid breaks and fragments
Chromatid Exchange Figures	include quadriradials, triradials, and complex rearrangements
Chromosome Breaks	include breaks and acentric fragments (Br); Dic, dicentric chromosome
Ex	exchange
Mitotic Index	(cells in mitosis / # cells scored), expressed as a percentage
Mitotic Inhibition	[(control mitotic index – treatment mitotic index)/control mitotic index], expressed as a percentage
SD	standard deviation
Severely Damaged Cells	includes cells with one or more pulverized chromosome and cells with 10 or more structural aberrations

Note: All calculated values are rounded.

Table 1
Preliminary toxicity test using H-27529 in the absence or presence of exogenous metabolic activation

	4 Hours						20 Hours		
	S9-			S9+			S9-		
Treatment[a] (µg/mL)	Cell Count (cells/ml x 10^6)	Cell Growth Index (%)	Cell Growth Inhibition[b] (%)	Cell Count (cells/ml x 10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)	Cell Count (cells/ml x 10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)
Vehicle[c]	0.39	100	NA	0.40	100	NA	0.37	100	NA
0.3	0.42	108	-8	0.38	95	5	0.45	122	-22
1	0.39	100	0	0.36	90	10	0.47	127	-27
3	0.43	110	-10	0.34	85	15	0.47	127	-27
10	0.44	113	-13	0.38	95	5	0.47	127	-27
30	0.44	113	-13	0.37	93	8	0.48	130	-30
100	0.38	97	3	0.32	80	20	0.48	130	-30
300	0.39	100	0	0.39	98	3	0.48	130	-30
1000	0.44	113	-13	0.42	105	-5	0.34	92	8
3471[d]	0.36	92	8	0.49	123	-23	0.15	41	59

[a]CHO cells were treated at 37ºC.
[b]Negative value indicates growth increase and therefore no growth inhibition.
[c]Water
[d]Equivalent to a 10 mM concentration.

Table 2
Concurrent toxicity test using H-27529 in the absence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	**Flask**	**Cell Count (cells/ml x 10^6)**	**Cell Growth Index (%)**	**Cell Growth Inhibition (%)**	**Mitotic Index (%)**	**Mitotic Inhibition[b] (%)**
Vehicle[c]	A B	0.66 0.65	100	NA	14.2	NA
100	A B	0.66 0.56	93	7	13.7	3.5
500	A B	0.59 0.62	92	8	13.2	7.0
1000	A B	0.57 0.60	89	11	15.6	-9.9
2500	A B	0.39 0.51	69	31	15.2	-7.0
3471[d]	A B	0.45 0.44	68	32	9.5	33.1
MMC 0.2	A B	0.41 0.48	68	32	13.0	8.5
MMC 0.4	A B	0.47 0.47	72	28	10.5	26.1

[a]CHO cells were treated at 37ºC.
[b]Negative value indicates mitotic increase and therefore no mitotic inhibition.
[c]Water
[d]Equivalent to a 10 mM concentration.

Table 3
Concurrent toxicity test using H-27529 in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	**Flask**	**Cell Count (cells/ml x 10^6)**	**Cell Growth Index (%)**	**Cell Growth Inhibition[b] (%)**	**Mitotic Index (%)**	**Mitotic Inhibition (%)**
Vehicle[c]	A B	0.45 0.51	100	NA	13.5	NA
100	A B	0.51 0.55	110	-10	7.1	47.4
500	A B	0.48 0.54	106	-6	11.6	14.1
1000	A B	0.62 0.49	116	-16	12.6	6.7
2500	A B	0.41 0.50	95	5	11.7	13.3
3471[d]	A B	0.48 0.49	101	-1	10.2	24.4
CP 5	A B	0.35 0.34	72	28	6.0	55.6
CP 10	A B	0.24 0.29	55	45	5.2	61.5

[a]CHO cells were treated at 37ºC.
[b]Negative value indicates growth increase and therefore no growth inhibition.
[c]Water
[d]Equivalent to a 10 mM concentration.

Table 4
Concurrent toxicity test using H-27529 in the absence of exogenous metabolic activation (20-hour continuous treatment)

Treatment[a] (µg/mL)	**Flask**	**Cell Count (cells/ml x 10^6)**	**Cell Growth Index (%)**	**Cell Growth Inhibition (%)**	**Mitotic Index (%)**	**Mitotic Inhibition (%)**
Vehicle[b]	A B	0.77 0.74	100	NA	21.0	NA
100	A B	0.73 0.75	98	2	18.2	13.3
500	A B	0.65 0.67	87	13	11.2	46.7
1000	A B	0.55 0.54	72	28	7.6	63.8
2500	A B	0.38 0.38	50	50	0.5	97.6
3471[c]	A B	0.31 0.32	42	58	0.2	99.0
MMC 0.2	A B	0.49 0.57	70	30	8.6	59.0
MMC 0.4	A B	0.50 0.50	66	34	5.2	75.2

[a]CHO cells were treated at 37ºC.
[b]Water
[c]Equivalent to a 10 mM concentration.

Table 5
Cytogenetic analysis of CHO cells treated with H-27529 in the absence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	Flask	Mitotic Index (%)	Number of Cells Scored		% Aberrant Cells[b]		Total Number of Structural Aberrations					Severely Damaged Cells	Average Aberrations Per Cell
								Chromatid		Chromosome			
			Numerical	Structural	Numerical	Structural	Gaps	Br	Ex	Br	Ex		
Vehicle[c]	A	17.0	100	100	1	2	4	1	0	0	1	0	0.020
	B	11.4	100	100	3	0	10	0	0	0	0	0	0.000
1000	A	17.8	100	100	3	0	5	0	0	0	0	0	0.000
	B	13.4	100	100	3	1	4	0	1	0	0	0	0.010
2500	A	15.4	100	100	10	4	0	3	0	0	1	0	0.040
	B	15.0	100	100	4	1	6	1	0	0	0	0	0.010
3471[d]	A	9.2	100	100	13	0	6	0	0	0	0	0	0.000
	B	9.8	100	100	10	1	2	1	0	0	0	0	0.010
MMC 0.2	A	15.0	25	25	8	36	2	8	2	1	0	0	0.440
	B	11.0	25	25	4	40	1	8	2	1	0	0	0.440

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 10 mM concentration.

Endoreduplicate Analysis

Treatment (µg/mL)	Flask	Number of Cells Scored	Number of Polyploid Cells	Number of Endoreduplicated Cells	% Endoreduplicated Cells
Vehicle	A	100	1	0	0.0
	B	100	3	0	
1000	A	100	3	0	0.0
	B	100	3	0	
2500	A	100	10	4	3.0
	B	100	4	2	
3471	A	100	13	8	7.0
	B	100	10	6	
MMC 0.2	A	25	2	0	0.0
	B	25	1	0	

Table 6
Cytogenetic analysis of CHO cells treated with H-27529 in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	Slide	Mitotic Index (%)	Cell Scored		% Aberrant Cells[b]		Total Number of Structural Aberrations					Severely Damaged Cells	Average Aberrations Per Cell
								Chromatid		Chromosome			
			Numerical	Structural	Numerical	Structural	Gaps	Br	Ex	Br	Ex		
Vehicle[c]	A	13.4	100	100	1	3	1	1	0	1	1	0	0.030
	B	13.6	100	100	1	1	6	2	0	0	0	0	0.020
1000	A	11.8	100	100	5	1	4	1	0	0	0	0	0.010
	B	13.4	100	100	1	0	4	0	0	0	0	0	0.000
2500	A	12.4	100	100	13	3	7	2	0	1	0	0	0.030
	B	11.0	100	100	10	0	4	0	0	0	0	0	0.000
3471[d]	A	11.2	100	100	15	3	4	0	2	1	0	0	0.030
	B	9.2	100	100	18	1	4	1	0	0	0	0	0.010
CP 5	A	7.8	100	100	0	16	1	15	5	0	1	0	0.210
	B	4.2	100	100	1	17	3	14	6	2	0	0	0.220

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 10 mM concentration.

Endoreduplicate Analysis

Treatment (µg/mL)	Flask	Number of Cells Scored	Number of Polyploid Cells	Number of Endoreduplicated Cells	% Endoreduplicated Cells
Vehicle	A	100	1	1	0.5
	B	100	1	0	
1000	A	100	5	3	2.0
	B	100	1	1	
2500	A	100	13	8	8.0
	B	100	10	8	
3471	A	100	15	13	13.5
	B	100	18	14	
CP 5	A	100	0	0	0.0
	B	100	1	0	

Table 7
Cytogenetic analysis of CHO cells treated with H-27529 in the absence of exogenous metabolic activation (20-hour continuous treatment)

Treatment[a] (µg/mL)	Slide	Mitotic Index (%)	Cell Scored		% Aberrant Cells[b]		Total Number of Structural Aberrations					Severely Damaged Cells	Average Aberrations Per Cell
								Chromatid		Chromosome			
			Numerical	Structural	Numerical	Structural	Gaps	Br	Ex	Br	Ex		
Vehicle[c]	A	14.2	100	100	4	3	9	2	0	1	0	0	0.030
	B	27.8	100	100	1	1	7	1	0	0	0	0	0.010
100	A	15.6	100	100	7	0	2	0	0	0	0	0	0.000
	B	20.8	100	100	3	0	4	0	0	0	0	0	0.000
500	A	11.4	100	100	3	1	11	1	0	0	0	0	0.010
	B	11.0	100	100	2	0	5	0	0	0	0	0	0.000
1000[d]	A	5.4	100	100	5	0	9	0	0	0	0	0	0.000
	B	9.8	100	100	3	1	3	1	0	0	0	0	0.010
MMC 0.2	A	9.6	25	25	4	40	1	12	3	3	0	0	0.720
	B	7.6	25	25	0	40	0	11	1	1	0	0	0.520

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 2.88 mM concentration.

Endoreduplicate Analysis

Treatment (µg/mL)	Flask	Number of Cells Scored	Number of Polyploid Cells	Number of Endoreduplicated Cells	% Endoreduplicated Cells
Vehicle	A	100	4	0	0.0
	B	100	1	0	
100	A	100	7	0	0.0
	B	100	3	0	
500	A	100	3	0	0.0
	B	100	2	0	
1000	A	100	5	1	0.5
	B	100	3	0	
MMC 0.2	A	25	1	0	0.0
	B	25	0	0	

Table 8
Summary

Treatment[a] µg/mL	S9 Activation	Treatment Time	Mitotic Index (%)	Cells Scored		Aberrations Per Cell		Cells with Aberrations[b]		
				Numerical	Structural	Mean	SD	Numerical (%)	Structural (%)	Endoreduplication (%)
Vehicle[c]	-S9	4	14.2	200	200	0.010	0.014	2.0	1.0	0.0
1000	-S9	4	15.6	200	200	0.005	0.007	3.0	0.5	0.0
2500	-S9	4	15.2	200	200	0.025	0.021	7.0[e]	2.5	3.0
3471[d]	-S9	4	9.5	200	200	0.005	0.007	11.5[e,f]	0.5	7.0
MMC 0.2	-S9	4	13.0	50	50	0.440	0.000	6.0	38.0[f]	0.0
Vehicle	+S9	4	13.5	200	200	0.025	0.007	1.0	2.0	0.5
1000	+S9	4	12.6	200	200	0.005	0.007	3.0	0.5	2.0
2500	+S9	4	11.7	200	200	0.015	0.021	11.5[e,f]	1.5	8.0
3471	+S9	4	10.2	200	200	0.020	0.014	16.5[e,f]	2.0	13.5
CP 5	+S9	4	6.0	200	200	0.215	0.007	0.5	16.5[f]	0.0
Vehicle	-S9	20	21.0	200	200	0.020	0.014	2.5	2.0	0.0
100	-S9	20	18.2	200	200	0.000	0.000	5.0	0.0	0.0
500	-S9	20	11.2	200	200	0.005	0.007	2.5	0.5	0.0
1000	-S9	20	7.6	200	200	0.005	0.007	4.0	0.5	0.5
MMC 0.2	-S9	20	8.6	50	50	0.620	0.141	2.0	40.0[f]	0.0

[a]CHO cells were treated at 37ºC.
[b]Excluding cells with only gaps.
[c]Water
[d]Equivalent to a 10 mM concentration.
[e]Statistically significant difference from control at $p < 0.05$ by Cochran-Armitage trend test.
[f]Statistically significant difference from control at $p < 0.05$ by Fisher's test.

APPENDICES

Appendix A
Historical Control Data

HISTORICAL CONTROL DATA[a]

Historical Values	Non-Activated Test System		S9-Activated Test System	
	Solvent Control (%)	Positive Control[b] (%)	Solvent Control (%)	Positive Control[c] (%)
Structural Chromosome Aberrations				
Mean	0.92	30.5	1.31	32.8
Standard Deviation	1.49	14.3	1.94	21.8
Range	0 – 5	10 – 51	0 – 6	7 – 68
Numerical Chromosome Aberrations				
Mean	1.21	0.14	1.63	0.29
Standard Deviation	1.39	0.38	1.90	0.49
Range	0 – 5	0 – 1	0 – 5	0 – 1

[a] Data are based on studies conducted 2002-2004. Data include all control solvents or diluents and metabolic activation systems based on Aroclor-induced rat liver S9.
[b] Mitomycin C (MMC)
[c] Cyclophosphamide (CP)